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TI .omega.-Amide and .omega.-amino acid derivatives of .alpha.-ketoglutaric acid and oxalacetic acids
AU Otani, Theodore T.; Meister, Alton
CS Natl. Cancer Inst., Bethesda, MD
SO J. Biol. Chem. (1957), 224, 137-48

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TI The mechanism and specificity of the glutamine-.alpha.-keto acid transamination-deamidation
AU Meister, Alton
CS Natl. Cancer Inst., Bethesda, MD
SO J. Biol. Chem. (1954), 210, 17-35

Preparation and enzymic reactions of the keto analogs of asparagine and glutamine
AU Meister, Alton
CS Natl. Inst. of Health, Bethesda, MD
SO J. Biol. Chem. (1953), 200, 571-89
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Irene Marx
Art Unit 1651
CM1 10-E-05,
Mail Box 11-B-01
703-308-2922

STUDIES ON THE MECHANISM AND SPECIFICITY OF THE GLUTAMINE-α-KETO ACID TRANSAMINATION-DEAMIDATION REACTION

By ALTON MEISTER

(From the National Cancer Institute, National Institutes of Health, Bethesda, Maryland)

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In previous reports (1, 2) the following transamination reaction, catalyzed by a purified rat liver preparation, was described:

(1) Glutamine + α-keto acid → α-amino acid + α-ketoglutaric acid + NH_3

Glutamine was not deamidated in the absence of an α-keto acid, and substitution of glutamine by glutamate resulted in a marked decrease or loss of transamination with most of the α-keto acids studied. The evidence indicated that deamidation did not precede transamination, suggesting the possibility that the α-keto analogue of glutamine (α-ketoglutaramic acid) might be an intermediate in the reaction. The finding that α-ketoglutaramate was deamidated to α-ketoglutarate by the enzyme preparation which catalyzed Reaction 1 was compatible with this hypothesis (3).

In an attempt to gain further information concerning the mechanism of the reaction, a number of glutamine analogues and related compounds, including α-methylglutamine, γ-methylglutamine,[1] homoglutamine, and several α-keto acids, were prepared and studied. It has now been found that γ-methylglutamine is appreciably active in transamination in this system, but that, in contrast to glutamine, no ammonia is formed. Furthermore, it has also been found that the α-keto analogue of γ-methylglutamine (α-keto-γ-methylglutaramic acid) is not deamidated by the enzyme preparation. This advantageous difference between the specificities of the transaminase and the amidase system has made possible the demonstration of α-keto-γ-methylglutaramic acid as a product of γ-methylglutamine-α-keto acid transamination. The evidence which has accumulated strongly suggests that Reaction 1 occurs by a two-step mechanism in which α-ketoglutaramic acid is an intermediate.

[1] In this paper, γ-methylglutamic acid and γ-methylglutamine refer to 2-amino-4-methylglutaric and 2-amino-4-methylglutaramic acids, respectively. The term γ-ethylglutamate (as well as γ-methylglutamate) has been used by some authors for the corresponding ω-ester, which will be designated here as glutamic acid-γ-ethyl ester.

EXPERIMENTAL

Materials and Methods

Enzyme Preparations—The lyophilized glutamine transaminase-deamidase preparation (2) and the purified α-keto acid-ω-amidase preparation (3) used in earlier studies were employed.

L-α-*Aminoadipamic Acid* (L-*Homoglutamine*)—25 gm. of DL-α-aminoadipic acid (4) were suspended in 500 ml. of ethanol, and the mixture was saturated with dry hydrogen chloride gas. After standing at room temperature for 1 hour, the clear solution was evaporated *in vacuo* to a syrup, which was dissolved in 100 ml. of water. The amino acid diester was converted to the carbobenzoxy derivative (5), which was obtained as a colorless syrup. The carbobenzoxy-DL-α-aminoadipic diethyl ester was dissolved in 500 ml. of methanol and the solution was saturated with ammonia gas at 0°. After standing at room temperature for 6 days, the solution was evaporated *in vacuo* to a colorless oil, which readily crystallized on cooling. After recrystallization from acetone-petroleum ether, 23 gm. (51 per cent) of carbobenzoxy-DL-α-aminoadipic acid diamide were obtained; m.p. 189°. Calculated for $C_{14}H_{19}O_4N_3$; C 57.3, H 6.5, N 14.3; found, C 57.0, H 6.5, N 14.1 per cent.[2]

8 gm. of papain (Nutritional Biochemicals Corporation) and 1 gm. of L-cysteine hydrochloride were dissolved in 400 ml. of water. After adjustment to pH 5 by addition of 2 N sodium hydroxide, the solution was filtered through glass wool and used immediately. 20 gm. of carbobenzoxy-DL-α-aminoadipic acid diamide were suspended in 300 ml. of the papain solution and incubated at 37° for 46 hours, at which time 46 per cent of the α-amide N was hydrolyzed.[3] The insoluble carbobenzoxy-D-α-aminoadipic acid diamide was removed by filtration, and the solution was evaporated *in vacuo* to dryness. The residue was extracted five times with 200 ml. portions of methanol, and the methanol extracts were combined, acidified to Congo red paper with hydrochloric acid, and evaporated to dryness. The crude product was recrystallized once from ethyl acetate and twice from ethanol, yielding 7 gm. (70 per cent based on the L form) of carbobenzoxy-L-α-aminoadipamic acid; m.p. 163°. Calculated for $C_{14}H_{18}O_5N_2$, C 57.1, H 6.2, N 9.5; found, C 56.8, H 6.2, N 9.7 per cent.

[2] The microanalyses were performed by Mr. Robert J. Koegel and Dr. William C. Alford.

[3] The course of the reaction was followed by determinations of ammonia. When the papain solution was incubated without substrate for longer than 1 day, considerable ammonia formation was observed; it was therefore difficult to be certain as to whether the reaction ceased when exactly one-half of the α-amide was hydrolyzed. The possibility that small amounts of the D isomer were hydrolyzed cannot be excluded.

2 gm. of the carbobenzoxy compound were dissolved in 200 ml. of methanol containing 0.2 ml. of glacial acetic acid and hydrogenated with palladium catalyst. After crystallization from water-ethanol, 0.93 gm. (85 per cent) of L-α-aminoadipamic acid was obtained. Calculated for $C_6H_{10}O_3N_2$: C 45.0, H 7.6, N 17.5; found, C 44.7, H 7.6, N 17.4 per cent. $[\alpha]_D^{20}$ +2.6° (5.47 per cent in water); $[\alpha]_D^{20}$ +21.0° (1.71 per cent in 1 N hydrochloric acid).

The determinations of optical rotation which were carried out in hydrochloric acid were completed within 10 or 15 minutes of the time of preparation of the solution; less than 1 per cent hydrolysis occurred under these conditions. The specific optical rotations at 20° for L- and D-glutamine in 1.00 N hydrochloric acid were +31.8° (1.31 per cent) and −31.6° (1.36 per cent), respectively. The corresponding values in water were +6.3° (4.14 per cent) and −6.4° (3.44 per cent). The latter values are in close agreement with those reported by Fruton (5) for natural and synthetic samples of L-glutamine.

Acid Hydrolysis of L-α-*Aminoadipamic Acid*—A solution of 0.5 gm. of L-α-aminoadipamic acid in 20 ml. of N hydrochloric acid was boiled under reflux for 1 hour. After evaporation *in vacuo* to dryness, the residue was dissolved in 10 ml. of water, the pH was adjusted to 3.2 with pyridine, and the solution placed at 5° for 16 hours. The crystalline precipitate was recrystallized from hot water, yielding 368 mg. (73 per cent) of L-α-aminoadipic acid. Calculated, N 8.7; found, 8.8 per cent. $[\alpha]_D^{20}$ +24.7° (2.29 per cent in 5 N hydrochloric acid).

L- *and* D-α-*Aminoadipic Acid*—The isomers were prepared by enzymatic resolution (6)[4] of DL-α-aminoadipic acid (4). $[\alpha]_D^{20}$ (2 per cent in 5 N hydrochloric acid) for the L and D forms, respectively, was +25.0° and −25.0° (+25.0°, −25.0° (6)).

L-α-*Aminoadipic Acid-δ-Ethyl Ester*—2 gm. of L-α-aminoadipic acid were dissolved in 20 ml. of ethanol containing 2 gm. of dry hydrogen chloride. The solution was cooled in ice and sufficient triethylamine was added to neutralize the acid. After standing at 5° for 16 hours, the crystalline ester was filtered and recrystallized from water-ethanol; yield 1.8 gm. (77 per cent). Calculated for $C_8H_{15}O_4N$, C 50.8, H 8.0, N 7.4; found, C 50.7, H 8.1, N 7.6 per cent. The product was homogeneous on paper chromatography in five different solvent systems, contained no α-aminoadipic acid, and was quantitatively oxidized by the L-amino acid oxidase of rattlesnake venom.

D-α-*Aminoadipamic Acid* (D-*Homoglutamine*)—5 gm. of D-α-aminoadipic acid were dissolved in 50 ml. of ethanol containing 5 gm. of hydrogen chlo-

[4] The author is indebted to Dr. Sanford M. Birnbaum for the acylase I preparation used for the resolution.

ride, and the solution was evaporated *in vacuo* to dryness. The residue was taken up in 50 ml. of water and neutralized by addition of solid sodium bicarbonate. 24 gm. of sodium bicarbonate and 22 gm. of carbobenzoxy-chloride were added in three equal portions with vigorous mechanical stirring at 0°. Stirring was continued for 1 hour, after which the solution was extracted twice with 100 ml. of ether. The aqueous layer was acidified with hydrochloric acid and extracted six times with 100 ml. of ether. The ether extracts were combined, dried over sodium sulfate, and evaporated. The residue (1.5 gm.) was dissolved in 50 ml. of 28 per cent aqueous ammonia and allowed to stand at room temperature for 18 hours. After evaporation *in vacuo* to dryness, the residue was dissolved in 50 ml. of methanol containing 0.5 ml. of glacial acetic acid and was hydrogenated with palladium catalyst. The product contained about 5 per cent of an impurity which appeared to be α-aminoadipic acid as determined by paper chromatography. This was removed by chromatography on Amberlite XE-64, as described below. The yield of pure material was 0.45 gm. (9 per cent).[5] Calculated for $C_6H_{12}O_3N_2$, C 45.0, H 7.6, N 17.5; found, C 45.2, H 7.8, N 17.7 per cent. $[\alpha]_D^{20}$ −2.7° (6.38 per cent in water); $[\alpha]_D^{20}$ −21.4° (1.75 per cent in 1 N hydrochloric acid). The product was at least 99.5 per cent optically pure, as determined by means of rattlesnake L-amino acid oxidase (7). The values of optical rotation for L-α-aminoadipamic acid are nearly equal in magnitude to those obtained for the D isomer, which was prepared from optically pure D-α-aminoadipic acid. This observation and the finding that acid hydrolysis of the L α-aminoadipamic acid preparation yielded α-aminoadipic acid, whose rotation was almost identical with that of optically pure L-α-aminoadipic acid, suggest that the L isomer obtained by the papain procedure represents a product of relatively high optical purity.

Sodium α-Ketoadipamate—This keto acid was prepared by enzymatic oxidation of 2.043 gm. of L-α-aminoadipamic acid with 950 mg. of *Crotalus adamanteus* venom, according to the general procedure for the preparation of α-keto acids previously described (8). The oxidation was carried out in a volume of 40 ml. and was complete in 24 hours. The yield of keto acid, obtained as the sodium salt, was 1.8 gm. (77 per cent).[6] Calculated, for

[5] The low yield is apparently due to failure of the carbobenzoxylation reaction to proceed beyond about 20 per cent of theory. Shaking at 5° for 24 hours with an 8-fold excess of reagent (with magnesium oxide or sodium bicarbonate) did not improve the yield. On the other hand, we have invariably obtained carbobenzoxy-L-glutamic acid-γ-ethyl ester in 50 to 75 per cent yield, using the procedure described in the text.

[6] Addition of 2,4-dinitrophenylhydrazine (1 per cent in 2 N hydrochloric acid) to a solution of the keto acid resulted in an immediate crystalline precipitate. On the basis of nitrogen analysis and paper chromatographic study, these preparations contained between 10 and 20 per cent of the hydrazone of the corresponding α-ketodicarboxylic acid, formed by hydrolysis during preparation of the derivative.

$C_6H_8O_4NNa$, C 39.8, H 4.5, N 7.7, Na 12.7; found, C 40.2, H 4.6, N 7.5, Na 12.5 per cent. After refluxing the keto acid in 2 N hydrochloric acid for 1 hour, stoichiometric quantities of ammonia were obtained, and α-ketoadipic acid was isolated from the hydrolysate as the 2,4-dinitrophenylhydrazone. After recrystallization from hot water, the melting point was 208°, and a mixed melting point with an authentic sample of the hydrazone, prepared from α-ketoadipic acid synthesized according to Gault (9), showed no depression.

DL-*Piperidonecarboxylic Acid*—4 gm. of DL-α-aminoadipic acid were dissolved in 100 ml. of water and refluxed for 3 hours. The product was separated from unchanged amino acid and crystallized as described for the D isomer (6). The yield of DL-piperidonecarboxylic acid was 2.2 gm., representing a yield of 82 per cent, since 1 gm. of DL-α-aminoadipic acid was recovered. Calculated for $C_6H_9O_3N$, C 50.3, H 6.3, N 9.8; found, C 50.0, H 6.5, N 9.8 per cent.

DL-α-*Amino-δ-N-methyladipamic Acid*—2 gm. of DL-piperidonecarboxylic acid were dissolved in 100 ml. of 30 per cent aqueous methylamine, and the solution was placed in a pressure bottle at 37° for 6 days. The product was obtained by evaporation of the solution, followed by crystallization from water-ethanol; yield 1.5 gm. (62 per cent). Calculated, for $C_7H_{14}O_3N_2$, C 48.5, H 8.1, N 16.1; found, C 48.0, H 8.0, N 16.0 per cent.

Barium α-Keto-N-methyladipamate—Oxidation of 2.162 gm. of DL-α-amino-δ-*N*-methyladipamic acid was carried out in a volume of 40 ml. with 1 gm. of *C. adamanteus* venom; the reaction was complete in 27 hours. The keto acid was isolated as described (8), yielding 2 gm. of the barium salt (67 per cent).[6] Calculated for $C_7H_{10}O_4NBa_{\frac{1}{2}}$, C 34.9, H 4.2, N 5.8, Ba 28.6; found, C 34.8, H 4.5, N 5.7, Ba 28.3 per cent.

L-γ-*Glutamyldimethylamide*—4 gm. of carbobenzoxy-L-γ-glutamylhydrazide were converted to the azide in chloroform (10) and added to 1.8 gm. of anhydrous dimethylamine in 100 ml. of chloroform. After standing at room temperature for 18 hours, the chloroform solution was washed successively with N hydrochloric acid and water, dried over sodium sulfate, and evaporated to a colorless oil. This was dissolved in 100 ml. of methanol containing 0.2 ml. of glacial acetic acid, and hydrogenated with palladium catalyst. The product was crystallized from water-ethanol; yield 0.71 gm. (30 per cent based on the hydrazide). Calculated for $C_7H_{14}O_3N_2$, C 48.5, H 8.1, N 16.1; found, C 48.0, H 8.1, N 16.3 per cent.

Barium α-Keto-N-dimethylglutaramate—This compound was prepared by oxidation of 1.357 gm. of L-γ-glutamyldimethylamide with 1 gm. of venom in a volume of 24 ml. The oxidation (8) was complete in 9 hours; the yield of barium salt was 1 gm. (53 per cent).[6] Calculated for $C_7H_{10}O_4NBa_{\frac{1}{2}}$, C 34.9, H 4.2, N 5.8, Ba 28.5; found, C 35.1, H 4.6, N 5.7, Ba 28.8 per cent.

*dl-γ-Methyl-*DL*-glutamic Acid*—This was obtained by condensation of

methyl methacrylate and diethyl acetamidomalonate, as described by Done and Fowden (11). The amino acid was isolated from the acid hydrolysate of the condensation product as follows. After repeated evaporation *in vacuo* to remove excess hydrochloric acid, the residue was taken up in water and adjusted to pH 3.2 with saturated lithium hydroxide. Several volumes of ethanol were added and the free amino acid was crystallized on cooling in ice. After recrystallization from water-acetone, the product was obtained in 69 per cent yield (based on diethyl acetamidomalonate). Calculated for $C_6H_{11}O_4N$, C 44.7, H 6.9, N 8.7; found, C 44.6, H 6.9, N 8.5 per cent. It is probable that the product represents a mixture of four stereoisomers.

*dl-γ-Methyl-*L*-glutamine*—Carbobenzoxy-*dl*-γ-methyl-DL-glutamic acid diamide was prepared from the residue obtained by evaporation of the hydrolyzed condensation product, as described above for the corresponding α-aminoadipic acid derivative and recrystallized from ethanol; yield 80 per cent; m.p. 190 198°. Calculated for $C_{14}H_{19}O_4N_3$, C 57.3, H 6.5, N 14.3; found, C 57.1, H 6.2, N 14.1 per cent. 25 gm. of the diamide were suspended in 300 ml. of papain solution, prepared as described above, at pH 5, and incubated for 24 hours, at which time 50 per cent of the α-amide N was hydrolyzed.[3] The insoluble carbobenzoxy-*dl*-γ-methyl-D-glutamic acid diamide was removed by filtration, and the solution was worked up as described for the corresponding α-aminoadipic acid derivative. The carbobenzoxy-*dl*-γ-methyl-L-glutamine was obtained as a thick syrup, which was hydrogenated in methanol-water with palladium catalyst. After crystallization from water-ethanol, 3.9 gm. (57 per cent, based on the L diamide) of *dl*-γ-methyl-L-glutamine were obtained. Calculated for $C_6H_{12}O_3N_2$, C 45.0, H 7.5, N 17.6; found, C 44.8, H 7.4, N 17.4 per cent. $[\alpha]_D^{20}$ + 30.6° (1.27 per cent in 1 N hydrochloric acid; the determination was carried out within 10 minutes of preparation of the solution).

0.4 gm. of the γ-methylglutamine preparation was dissolved in 10 ml. of 2 N hydrochloric acid and refluxed for 1 hour. The γ-methylglutamic acid was isolated as described below; N, calculated 8.7, found 8.6 per cent. $[\alpha]_D^{20}$ +31.3° (1.15 per cent in 5 N hydrochloric acid).

The γ-methylglutamine preparation would contain about 10 per cent of the D enantiomorph, on the basis of comparison of the magnitude of the rotations of the γ-methylglutamic acid obtained from it by acid hydrolysis and that of the γ-methylglutamic acid derived from carbobenzoxy-*dl*-γ-methyl-D-glutamic acid diamide. The presence of D isomer in the γ-methylglutamine preparation could be explained by hydrolysis of carbobenzoxy-*dl*-γ-methyl-D-glutamic acid diamide by the papain preparation. Oxidation of the γ-methylglutamine preparation by rattlesnake L-amino acid oxidase proceeded to 80 per cent of completion, as judged by oxy-

gen consumption and ammonia formation; optically pure L-glutamine was oxidized to 90 per cent of theory under these conditions. It is also possible that the differences in optical rotation may be related to fractionation of the diastereoisomers.

dl-γ-Methyl-D-glutamic Acid—The crude carbobenzoxy-*dl*-γ-methyl-D-glutamic acid diamide obtained above was recrystallized twice from ethanol; yield 7 gm. (56 per cent); m.p. 210°. Calculated for $C_{14}H_{19}O_4N_3$, C 57.3, H 6.5, N 14.3; found, C 57.0, H 6.3, N 14.6 per cent. 2 gm. of carbobenzoxy-*dl*-γ-methyl-D-glutamic acid diamide were dissolved in 100 ml. of 3 N hydrochloric acid and hydrogenated with palladium catalyst. After the reaction was complete, the catalyst was removed and the solution was refluxed for 2 hours. The excess hydrochloric acid was removed by repeated evaporation *in vacuo*, and the residue was dissolved in 10 ml. of water. The pH was brought to 3.2 with saturated lithium hydroxide, and the product was crystallized by addition of 30 ml. of ethanol and cooling at −20° for 18 hours. After two recrystallizations from water-ethanol, 0.55 gm. (50 per cent) of *dl*-γ-methyl-D-glutamic acid was obtained; calculated, 8.7 N; found, 8.6 per cent. $[\alpha]_D^{20}$ −40.1° (1.87 per cent in 5 N hydrochloric acid).

Barium α-Keto-dl-γ-methylglutaramate—1 gm. of *dl*-γ-methyl-L-glutamine was oxidatively deaminated with 1 gm. of venom in a final volume of 35 ml., as described (8). The reaction was complete in 3 hours, and the keto acid was isolated as the barium salt; yield 800 mg. (56 per cent). It was desirable to complete the oxidation in a few hours because of the relatively rapid spontaneous deamidation of the substrate. Calculated for $C_6H_8O_4NBa_{\frac{1}{2}}$, C 31.8, H 3.6, N 6.2, Ba 30.6; found, C 31.6, H 3.4, N 6.1, Ba 30.3 per cent. Addition of 1 per cent 2,4-dinitrophenylhydrazine in 2 N hydrochloric acid to a solution of the keto acid resulted in the slow formation of a crystalline hydrazone over a period of several days; this product contained about 10 per cent of the hydrazone of α-keto-*dl*-γ-methylglutaric acid, as determined by paper chromatography. The latter derivative was prepared by addition of 2,4-dinitrophenylhydrazine to a solution of α-keto-*dl*-γ-methylglutaramic acid in 2 N hydrochloric acid which had been refluxed for 3 hours. The hydrazone was crystallized from ethyl acetate-petroleum ether. Calculated for $C_{12}H_{12}O_8N_4$, N 16.5; found, 16.5 per cent; m.p. 180°.

α-Methyl-DL-glutamine—Carbobenzoxy-α-methyl-DL-glutamic acid diamide[7] was prepared as described above for the corresponding α-aminoadipic acid derivative; yield 50 per cent; m.p. 158°. Calculated for $C_{14}H_{19}O_4N_3$, C 57.3, H 6.5, N 14.3; found, C 57.4, H 6.5, N 14.2 per cent. Since this compound was resistant to attack by papain, α-methyl-DL-glutamine

[7] The author is indebted to Dr. Karl Pfister of Merck and Company, Inc., for a very generous quantity of α-methyl-DL-glutamic acid.

was prepared via the carbobenzoxy-ω-ester as described above for D-α-aminoadipamic acid.[8] The product contained about 10 per cent of α-methyl-DL-glutamic acid, which was removed by chromatography as described below. After crystallization from water-acetone, a 10 per cent yield[5] of pure α-methyl-DL-glutamine was obtained. Calculated for $C_6H_{12}O_3N_2$, C 45.0, H 7.6, N 17.5; found, C 45.3, H 7.9, N 17.4 per cent.

Chromatographic Purification of Glutamine and Related Compounds—Glutamine preparations, whether isolated or synthetic, have frequently been found to contain glutamic acid in quantities varying from traces to as much as 10 per cent. Although crystallization from water is useful for removal of asparagine and arginine, the concentration of glutamic acid is not appreciably affected. We have therefore employed the following procedure for the purification of glutamine. 30 gm. of glutamine dissolved in 1 liter of water were added to the top of a well washed Amberlite XE-64 column (height 173 cm., diameter 10 cm.). The ion exchange resin, obtained from the Rohm and Haas Company, was washed thoroughly by decantation to remove fine particles, and was then washed successively with 6 N sodium hydroxide, water, 6 N hydrochloric acid, and finally with water, until the wash was chloride-free. Chromatography was carried out in a cold room at 5° with water as the eluting agent. Fractions of approximately 100 ml. were collected every 30 minutes. Glutamic acid appeared in the effluent after about 13 liters had flowed through the column, and glutamine (free of glutamic acid) appeared in the effluent in Fractions 150 to 160 (corresponding to the 15th liter). The fractions containing glutamine were combined and evaporated *in vacuo* to low bulk. The glutamine was crystallized from warm water (50°) by addition of 3 volumes of ethanol. The final product was free of glutamic acid, as determined by paper chromatography and by enzymatic methods (12).

A small scale adaptation of this procedure, which was used in order to remove α-methylglutamic and α-aminoadipic acids from α-methylglutamine and homoglutamine, respectively, in the present investigation, was carried out on a column 168 cm. by 2.5 cm., with a flow rate of 10 to 30 ml. per hour.

Other Compounds—L-γ-Glutamylmethylamide (13), L-γ-glutamylethylamide (13), L-glutamic acid-γ-ethyl ester (14), L-γ-glutamylhydroxamic acid (12), L-γ-glutamylhydrazide (10), L-γ-glutamylglycine (10), and L-γ-glutamylglycine ethyl ester (10) were prepared as described. The preparation of the other α-keto acids employed in this investigation is given else-

[8] Chloroacetyl-α-methyl DL glutamic acid (m.p. 163°) was not susceptible to the action of acylase I, as determined by the Van Slyke nitrous acid procedure, kindly carried out for us by Dr. Sanford M. Birnbaum; enzymatic resolution with acylase I was therefore not attempted.

where (8). The author is indebted to Dr. Jesse P. Greenstein for generous amounts of L-isoglutamine and L-isoasparagine.

Methods—L-Glutamine and L-glutamic acid (15, 16), pyruvate, glyoxylate, and α-ketobutyrate (17), α-ketoglutarate (1), and L-phenylalanine (18) were determined as described. Ascending paper chromatography was carried out on Whatman No. 4 paper with the following solvent systems: (*a*) formic acid, water, tertiary butanol (15:15:70); (*b*) phenol saturated with 10 per cent aqueous sodium carbonate; (*c*) 77 per cent ethanol; (*d*) pyridine, water, methanol (4:20:80); (*e*) methylethyl ketone, tertiary butanol, formic acid, water (16:16:0.1:3.9); (*f*) pyridine, tertiary amyl alcohol, water (35:35:30); (*g*) *n*-butanol, water, ethanol (50:40:10; upper layer (19)); (*h*) *n*-propanol, 28 per cent aqueous ammonia, water (60:30:10). Solvents (*a*) to (*e*) inclusive were used for amino acids, and chromatograms of the hydrazones were developed with solvents (*b*), (*c*), (*f*), (*g*), and (*h*).

Determination of amino acids by densitometric measurements of the corresponding spots on paper chromatograms was carried out by a procedure similar to that described by Block *et al.* (20).[9] Determinations of glycine, alanine, and tyrosine were performed with chromatograms developed in solvent (*a*); those of α-aminobutyric acid, norvaline, and norleucine were carried out with chromatograms developed with solvent (*c*), and chromatograms developed with solvents (*a*) and (*c*) were found suitable for determination of leucine, methionine, and phenylalanine. Where independent checks were available (*e.g.*, by use of the decarboxylase method for phenylalanine and tyrosine, and by determinations of the disappearance of α-keto acids (Table III)), good agreement was observed.

Stability of Amino Acid ω-Amides and Related Compounds—The well known tendency to ring closure exhibited by glutamine is apparently shared by α-methylglutamine, γ-methylglutamine, and homoglutamine. Gilbert, Price, and Greenstein (21) observed non-enzymatic conversion of glutamine to pyrrolidonecarboxylic acid and ammonia in the presence of phosphate (and certain other anions) at pH 8, and concluded that this reaction proceeded by a mechanism different from that involved in the phosphate-dependent enzymatic hydrolysis of glutamine. In the present investigation, the phosphate-catalyzed non-enzymatic deamidation reaction was studied with glutamine, homoglutamine, α-methylglutamine, and γ-methylglutamine. The deamidation of glutamine and α-methylglutamine occurred at approximately the same rate, while the reaction was appreciably faster with homoglutamine, a finding compatible with the expected greater tendency of this compound to cyclize. Arsenate and bicarbonate also catalyzed the non-enzymatic deamidation of homoglutamine, whereas chloride, sulfate,

[9] The author is indebted to Dr. Herbert A. Sober for advice and assistance in connection with this procedure.

and pyruvate did not (*cf.* Gilbert *et al.* (21)). It is interesting that γ-methylglutamine was deamidated considerably more rapidly than homoglutamine or glutamine (Fig. 1). A number of other compounds were studied in this system, and the reaction was followed semiquantitatively by means of paper chromatography. γ-Glutamylhydroxamic acid and γ-glutamylhydrazide disappeared at rates of approximately the same order of magnitude as observed with glutamine. On the other hand, glutamate, γ-glutamylmethylamide, γ-glutamyldimethylamide, γ-glutamylethylamide, γ-glutamylglycine, α-amino-δ-*N*-methyladipamic acid, and asparagine were

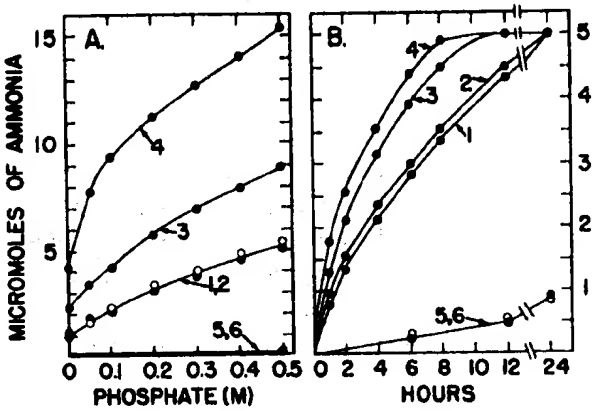


FIG. 1. Non-enzymatic deamidation of α-amino acid-ω-amides. Curve 1, L-glutamine; Curve 2, α-methyl-DL-glutamine; Curve 3, L-homoglutamine; Curve 4, *dl*-γ-methyl-L-glutamine; Curve 5, L-isoglutamine; Curve 6, L-asparagine. (*A*) Deamidation in the presence of potassium phosphate, pH 7.8; incubated at 37° for 6 hours. The mixtures consisted of 0.5 ml. of 0.1 M Veronal buffer (pH 7.8) containing 20 μM of amide, and 0.5 ml. of potassium phosphate (pH 7.8). The abscissa represents the final concentration of phosphate. (*B*) Deamidation in N hydrochloric acid; 37°. 0.01 M solutions of the amides in N hydrochloric acid were incubated at 37°, and 0.5 ml. aliquots were removed for ammonia analysis at the indicated intervals.

not significantly affected by incubation in 0.5 M potassium phosphate at pH 7.8 for 3 days. After 3 days the phosphate-catalyzed deamidation of glutamine, α-methylglutamine, homoglutamine, and γ-methylglutamine described in Fig. 1 had gone virtually to completion, as judged by loss of the ninhydrin color reaction, and recovery of more than 90 per cent of the theoretical ammonia.

In N hydrochloric acid at 37°, α-methylglutamine, homoglutamine, and γ-methylglutamine were hydrolyzed at rates which were approximately 15, 50, and 80 per cent, respectively, greater than that observed with glutamine (Fig. 1). In N hydrochloric acid at 100°, greater than 90 per cent hydrolysis of each of the four amides was observed in 9 minutes, and 98 to 100 per cent hydrolysis occurred after 20 minutes at this temperature.

Acid hydrolysis did not lead to loss of the ninhydrin color reaction, and the corresponding α-aminodicarboxylic acids were isolated after acid hydrolysis of homoglutamine and γ-methylglutamine as described above.

Properties of α-Keto Acids—It was previously reported that α-ketoglutaramic acid and α-ketosuccinamic acid were capable of existing in two interconvertible forms (3). One of these (previously designated Form B) appeared to possess a reactive α-keto group as judged by ability to form readily a 2,4-dinitrophenylhydrazone, susceptibility to decarboxylation by hydrogen peroxide, and susceptibility to enzymatic attack by a purified liver amidase preparation. On the other hand, α-ketoglutaramic and α-ketosuccinamic acids were also obtained in a form (previously designated Form A) which yielded hydrazones at relatively slow rates, and which was resistant to peroxide and to the action of the amidase. α-Keto-*N*-methylglutaramic acid, prepared by the same procedure as α-ketoglutaramic acid, appeared to exist only in a form not exhibiting properties characteristic of a reactive α-keto group. On the other hand, three of the α-keto acids described in this report (α-ketoadipamic, α-keto-*N*-methyladipamic, and α-keto-*N*-dimethylglutaramic acids) reacted promptly with 2,4-dinitrophenylhydrazine, and were rapidly decarboxylated by hydrogen peroxide at pH 4.9. Similar findings have also been made for α-ketoglutaric acid-γ-ethyl ester (8). These α-keto acids do not exhibit a tendency to occur in forms not possessing reactive α-keto groups. The ability to form such nonreactive structures would appear to depend upon a chain length of 4 or 5 carbon atoms, and the presence of an ω-amide group in which at least 1 hydrogen is unsubstituted. It is of interest that the *N*-methyl derivative of α-ketoadipamic acid reacted as a typical α-keto acid, in contrast to the behavior of the corresponding glutaramic acid derivative. These observations are consistent with a cyclic formulation for the structures of the nonreactive forms; however, further investigation is necessary.

α-Keto-*dl*-γ-methylglutaramic acid exhibited properties similar to those of α-ketoglutaramic acid; *i.e.*, (*a*) neither acid was decarboxylated at pH 4.9 by hydrogen peroxide; (*b*) in strongly acid (N sulfuric acid) solution, both compounds were slowly decarboxylated by peroxide, while both were rapidly decarboxylated by ceric sulfate yielding stoichiometric quantities of carbon dioxide; (*c*) both compounds were rapidly decarboxylated by hydrogen peroxide in alkaline solution (*cf.* (3)); (*d*) α-ketoglutaramic and α-keto-*dl*-γ-methylglutaramic acids formed 2,4-dinitrophenylhydrazones relatively slowly; (*e*) neither compound was reduced at an appreciable rate by lactic dehydrogenase;[10] and (*f*) the rates of deamidation of these compounds in N hydrochloric acid at 100° were approximately the same.

[10] α-Keto-*N*-methyladipamate, α-ketoadipate, α-ketoadipamate, and α-keto-*dl*-γ-methylglutaramate were, like α-ketoglutaramate, reduced at negligible rates by the

Enzyme Studies

Specificity of Enzyme for Amino Donor—In earlier studies on the glutamine-α-keto acid reaction, it was found that L-isoglutamine, L-glutathione, L-glutamic acid-γ-ethyl ester, and L-γ-glutamylethylamide were incapable of replacing L-glutamine in transamination in this system, whereas L-γ-glutamylmethylamide was active in transamination with α-keto acids and was simultaneously converted to α-ketoglutarate and methylamine. These derivatives have been investigated again, together with a number of other γ-glutamyl derivatives and related compounds. For the poorly reacting α-amino donors, concentrations of enzyme between 2 and 5 times that usually employed with glutamine were used. With the exception of L-γ-glutamylmethylamide, *dl*-γ-methyl-L-glutamine, and a γ-methyleneglutamine preparation isolated from tulip,[11] none of these was appreciably active (Table I). These findings indicate the relatively high degree of specificity of the enzyme system for the α-amino group donor. The failure of the α-methylglutamic acid derivatives to transaminate is compatible with the currently accepted mechanism of transamination involving Schiff's base formation.

Specificity of Enzyme for α-Keto Acid—The relatively narrow specificity of the system with respect to the amino donor is in striking contrast to the wide variety of α-keto acids which have been found to be active. In previous reports, twenty-eight of the thirty-five α-keto acids studied in this system were active. The inactive α-keto acids included oxalacetic acid, several keto acids possessing one or no β-hydrogen atom, and keto acids which tend to cyclize (22). It is of interest that, although homoglutamine, its ω-*N*-methyl derivative, and L-γ-glutamyldimethylamide were incapable of replacing glutamine as the amino donor, the α-keto analogues of these compounds were aminated to yield the corresponding amino derivatives when incubated with glutamine in this system (Table II).

Separation of Transamination and Deamidation Steps—Despite considerable effort, we have been unsuccessful in separating an enzyme capable of catalyzing transamination between glutamine and α-keto acids from that which catalyzes the deamidation of α-ketoglutaramic acid. Such a separation might be expected to permit isolation of α-ketoglutaramic acid as a product of glutamine-α-keto acid transamination. Difficulty in these at-

lactic dehydrogenase system (17). α-Keto-*N*-dimethylglutaramate was reduced at a rate of 560×10^{-5} mole per minute, compared to a rate of $26{,}800 \times 10^{-5}$ for pyruvate. (The conditions of these determinations were as previously described (17).)

[11] These experiments were made possible by the generosity of Dr. F. C. Steward, who provided us with a sample of γ-methyleneglutamine. Through the kindness of Dr. L. Fowden, we have recently been able to study a sample of γ-methyleneglutamine isolated from the peanut plant. The results were in agreement with those observed with the amide obtained from the tulip bulb.

tempts arose from the fact that the transaminase activity is relatively unstable and that the enzyme preparations possess considerably more amidase than transaminase activity. An alternative approach to this problem was therefore made, in which a number of glutamine analogues were studied in

TABLE I

Specificity of Amino Donor

α-Amino acid	Transamination*	α-Amino acid	Transamination*
L-Glutamine†	(100)‡§‖	L-Homoglutamine†	<5
L-γ-Glutamylmethylamide¶	65§	L-Asparagine†	<5
L-γ-Glutamyldimethylamide	<5	L-Aspartic acid†	<5
L-γ-Glutamylethylamide¶	<5	L-Isoasparagine	<5
L-Glutamic acid-γ-ethyl ester¶	<5	L-α-Aminoadipic acid-δ-ethyl ester	<5
L-Glutathione	<5	DL-α-Amino-δ-*N*-methyladipamic acid	<5
L-γ-Glutamylhydroxamic acid	<5	α-Methyl-DL-glutamine	<5
L-γ-Glutamylhydrazide	<5	α-Methyl-DL-glutamic acid	<5
L-γ-Glutamylglycine	<5	*dl*-γ-Methyl-DL-glutamic acid†	<5
L-γ-Glutamylglycine-ethyl ester	<5	*dl*-γ-Methyl-L-glutamine	61‖
L-Glutamic acid†	13§	γ Methyleneglutamine (isolated from tulip)	75††
L-Isoglutamine**	10§		

* The reaction mixtures contained 20 μM of L-amino acid, 20 μM of sodium phenylpyruvate, and 30 to 180 mg. of enzyme in 1.5 ml. of 0.1 M Veronal buffer (pH 8.2); incubated at 37° for 1 to 3 hours. The values for transamination are given in terms of the activity observed with L-glutamine, which is arbitrarily designated as 100. Similar relative values were obtained in experiments in which phenylpyruvate was replaced by α-ketoisocaproate.

† The D isomer was studied and found to be inactive.

‡ Based on determinations of glutamine and glutamate.

§ Based on formation of α-ketoglutarate.

‖ Based on L-phenylalanine formation.

¶ The DL compound (40 μM) gave similar results.

** L-Isoglutamine was slowly deamidated under these conditions.

†† Semiquantitative estimation based on paper chromatographic study.

the hope of finding one which would participate in the transamination reaction, but whose α-keto analogue would not be susceptible to attack by the amidase. Of the several derivatives investigated, *dl*-γ-methyl-L-glutamine appeared to fulfil these requirements. This amide transaminated with α-keto acids at a rate of the same order of magnitude as that observed with L-glutamine. However, in contrast to glutamine, this substrate was not deamidated. The values for transamination with γ-methylglutamine were 50 to 80 per cent of those obtained with glutamine. No transamina-

tion was observed with either amide and four α-keto acids previously found to be inert in this system (Table III). Under similar conditions, appreciable transamination was observed between γ-methyleneglutamine and pyruvate and between this amide and α-ketoisocaproate; in these studies, also, no deamidation occurred.

Experiments in which α-keto-*dl*-γ-methylglutaramate was incubated at pH values of 7.5 to 9.0 with the purified α-keto acid-ω-amidase preparation, or with the glutamine transaminase-deamidase preparation, indicated that this α-keto acid-ω-amide, in contrast to α-ketoglutaramic acid, was not deamidated. These results strongly suggested that the α-keto analogues of γ-methylglutamine and γ-methyleneglutamine were formed in the course of

TABLE II

*Transamination between Glutamine and α-Keto Acid-ω-Amides**

α-Keto acid	Transamination,† μM	Deamidation, μM NH_3
α-Ketoadipamic	4.21	4.31
α-Keto-*N*-methyladipamic	1.66	2.01
α-Keto-*N*-dimethylglutaramic	4.46	4.64
α-Keto-γ-methylglutaramic	0.95	1.05
Pyruvic	6.02	6.21

* The reaction mixtures contained initially 10 μM of L-glutamine, 20 μM of α-keto acid, and 40 mg. of enzyme in 1 ml. of 0.1 M Veronal buffer (pH 8.2); incubated at 37° for 2 hours. An experiment with pyruvic acid was carried out for comparative purposes.

† Values for transamination were derived from determinations of glutamine disappearance. The formation of the corresponding α-amino acid-ω-amides was observed by paper chromatography.

the transamination reactions. Isolation of α-keto-γ-methyleneglutaramic acid could not be attempted because of the limited amount of γ-methyleneglutamine available. Formation of α-keto-*dl*-γ-methylglutaramic acid was demonstrated by chromatography of the corresponding 2,4-dinitrophenylhydrazone.

Identification of α-Keto-dl-γ-methylglutaramic Acid—A mixture containing 200 μM of γ-methylglutamine, 400 μM of sodium pyruvate and 150 mg. of enzyme in 1.1 ml. of 0.05 M Veronal buffer (pH 8.2) was incubated for 2 hours at 37°. 0.5 ml. of 0.4 M sodium acetate buffer (pH 4.9) was added, and the mixture was placed at 100° for 2 minutes. The coagulated protein was removed by centrifugation, and the supernatant solution was mixed with 0.5 ml. of 10 per cent hydrogen peroxide and incubated at 37° for 15 minutes. The peroxide was decomposed by addition of 1 drop of a crystalline catalase suspension, and the solution was lyophilized. The residue

was mixed with 3 ml. of warm 1 per cent 2,4-dinitrophenylhydrazine in 2 N hydrochloric acid and allowed to stand for 18 hours, after which the solution was extracted three times with 3 ml. of ethyl acetate. The extracts were combined and chromatographed together with extracts obtained from similar experiments in which pyruvate and γ-methylglutamine were separately omitted. With the complete system, formation of the 2,4-dini-

TABLE III

*Comparison of Transamination with L-Glutamine and dl-γ-Methyl-L-glutamine**

	L-Glutamine		*dl*-γ-Methyl-L-glutamine†
	Transamination, μM‡	Deamidation, μM NH_3	Transamination, μM‡
Pyruvic acid	9.6 (9.30)	9.51	6.9 (6.15)
α-Ketobutyric acid	8.7 (9.00)	9.25	6.5 (5.85)
α-Ketovaleric "	8.8	9.21	5.3
α-Ketoisovaleric acid	0	0	0
α-Ketocaproic acid	8.4	9.20	5.7
α-Ketoisocaproic acid	8.7	8.90	5.8
d-α-Keto-β-methylvaleric acid	0	0	0
β,β,β-Trimethylpyruvic acid	0	0	0
Phenylpyruvic acid	6.0	5.81	3.0
p-Hydroxyphenylpyruvic acid	5.0	6.01	3.8
α-Ketophenylacetic acid	0	0	0
Glyoxylic acid	8.9 (9.05)	9.20	6.8 (6.65)
α-Keto-γ-methiolbutyric acid	9.1	9.01	6 6

* The reaction mixtures contained initially 20 μM of α-keto acid, 10 μM of amino acid amide, and 20 mg. of enzyme in 0.3 ml. of 0.1 M Veronal buffer (pH 8.2); incubated for 2 hours at 37°.

† No deamidation was observed.

‡ Transamination values are based on determinations of the amino acids formed, and, in three cases, on determinations of keto acid disappearance. The values based on disappearance of keto acids are given in parentheses.

trophenylhydrazone of α-keto-*dl*-γ-methylglutaramic acid was demonstrated by chromatographic comparison with a sample of this derivative prepared from the authentic keto acid.

The treatment with peroxide simplified the chromatographic identification by destroying residual pyruvate; as noted above, α-keto-*dl*-γ-methylglutaramic acid is not decarboxylated under these conditions. The R_F values for α-keto-*dl*-γ-methylglutaramic acid 2,4-dinitrophenylhydrazone in solvents (*b*), (*c*), (*f*), (*g*), and (*h*) were, respectively, 0.55 to 0.60, 0.53 to 0.58, 0.68 to 0.75, 0.38 to 0.45, and 0.61 to 0.67. The corresponding values for α-keto-*dl*-γ-methylglutaric acid 2,4-dinitrophenylhydrazone were 0.80

to 0.87, 0.65 to 0.70, 0.77 to 0.82, 0.70 to 0.80, and 0.77 to 0.85. Small amounts of this hydrazone were also visualized on the chromatograms.

Formation of γ-Methylglutamine from α-Keto-γ-methylglutaramate—With equimolar concentrations of γ-methylglutamine and pyruvate, and other conditions as given in Table III, the reaction was found to cease at about 70 per cent of maximal transamination. On the other hand, in experiments in which α-keto-γ-methylglutaramate and alanine were incubated, only traces of γ-methylglutamine were formed, as determined by paper chromatography. Similar results were obtained when α-keto-γ-methylglutaramate was incubated with a number of other amino acids. The failure of the reverse reaction to reach the same equilibrium point under the experimental conditions employed may possibly be related to the tendency of the α-keto acid-ω-amide to exist in a non-reactive form. α-Keto-γ-methylglutaramate was aminated to a slight but measurable extent when incubated with glutamine (Table II), and similar results were obtained with the asparagine system with conditions previously described (2).

DISCUSSION

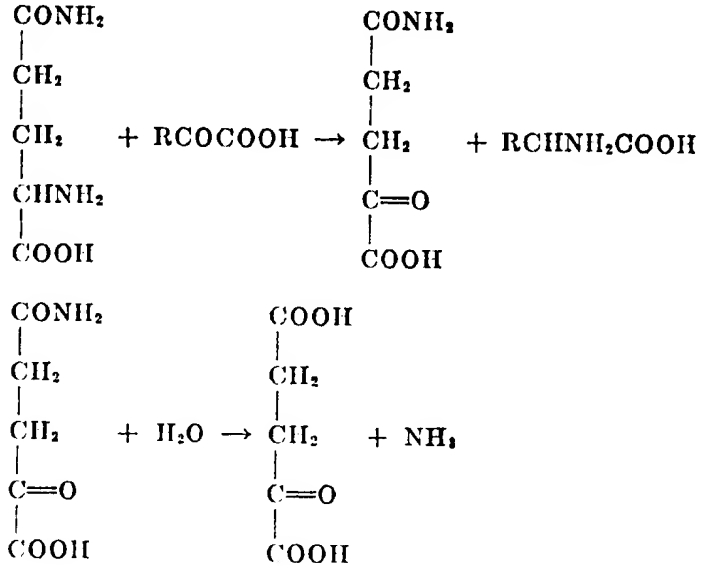
The studies on the specificity of the glutamine transaminase reaction indicate that, although a wide variety of α-keto acids is active in this system, only three of twenty-two compounds investigated can replace glutamine. The system therefore exhibits a considerably narrower specificity for the amino donor than for the amino group acceptor. It is of interest that several α-keto acids, which correspond to analogues of glutamine (*e.g.*, homoglutamine) which cannot replace glutamine as the amino donor, are active as amino group acceptors. The transaminase system appears to require an α-keto acid possessing at least two β-hydrogen atoms, and an amino acid-ω-amide with a chain of 5 carbon atoms with an unsubstituted (or monomethyl-substituted) ω-amide group.

A significant finding in relation to the mechanism of the reaction was that γ-methylglutamine and γ-methyleneglutamine were active in transamination, but did not yield ammonia. The experiments with γ-methylglutamine indicated that the corresponding α-keto acid-ω-amide accumulated, since it was not susceptible to the action of the amidase.[12] In contrast to

[12] The susceptibility of the α-methyl and γ-methyl derivatives of glutamic acid and glutamine was also studied in the following enzyme systems. (*a*) Rattlesnake venom L-amino acid oxidase oxidized *dl*-γ-methyl-L-glutamine and L-glutamine at approximately the same rate; *dl*-γ-methyl-DL-glutamic acid, α-methyl-DL-glutamine, and α-methyl-DL-glutamic acid were not attacked; (*b*) *C. welchii*, L-glutamic acid decarboxylase did not decarboxylate the methyl-substituted derivatives; (*c*) hog kidney D-amino acid oxidase did not oxidize the methyl-substituted derivatives; (*d*) *Escherichia coli* glutaminase hydrolyzed *dl*-γ-methyl-L-glutamine and α-methyl-DL-glutamine at about 1 and 25 per cent, respectively, of the rate observed with L-

the γ-methylglutamine-α-keto acid reaction, the glutamine-α-keto reaction proceeded virtually to completion with stoichiometric formation of ammonia. In the latter reaction the α-keto acid-ω-amidase system would be expected to drive the reaction to completion by removal of α-ketoglutaramate.

In view of these findings and considerations previously discussed, the accompanying reaction sequence for the glutamine-α-keto acid transamination-deamidation reaction appears highly probable. A similar sequence of enzymatic steps probably also occurs in the analogous asparagine-α-keto acid reaction, a conclusion which is supported by the demonstration of the reversal of the latter reaction, with the formation of asparagine from α-ketosuccinamic acid (23).



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SUMMARY

1. Although a wide variety of α-keto acids was active in the glutamine transaminase system, only three of twenty-two compounds investigated were capable of replacing glutamine as the amino donor. Homoglutamine, α-amino-δ-*N*-methyladipamic acid, and γ-glutamyldimethylamide were in-

glutamine. Studies with glutaminase prepared from rat kidney gave similar results; (*e*) suspensions of *E. coli* catalyzed transamination between *dl*-γ-methyl-DL-glutamic acid and phenylpyruvic acid, α-ketoisocaproic acid and α-keto-γ-methiolbutyric acid, at rates approximately 50 per cent of those observed with DL-glutamic acid.

active as amino donors, while the α-keto analogues of these amino acids were aminated when incubated with glutamine in this system.

2. γ-Methylglutamine and γ-methyleneglutamine were appreciably active in transamination with α-keto acids. However, in contrast to glutamine, no ammonia was formed with these amides. The evidence indicated that α-keto-γ-methylglutaramic acid accumulated in the reaction between γ-methylglutamine and α-keto acids. α-Keto-γ-methylglutaramic acid was not deamidated by the glutamine transaminase preparation or by a purified amidase preparation capable of rapidly hydrolyzing α-ketoglutaramic acid.

3. The present and previously reported evidence indicates that the glutamine-α-keto acid transamination-deamidation reaction involves two steps: (*a*) transamination between glutamine and keto acid yielding amino acid and α-ketoglutaramic acid, and (*b*) hydrolysis of α-ketoglutaramic acid to ammonia and α-ketoglutarate.

4. The preparation of L- and D-homoglutamine, L-α-aminoadipic acid-δ-ethyl ester, DL-α-amino-δ-*N*-methyladipamic acid, L-γ-glutamyldimethylamide, *dl*-γ-methyl-L-glutamine, *dl*-γ-methyl-D-glutamic acid, and α-methyl-DL-glutamine is described. The non-enzymatic deamidation of glutamine, homoglutamine, α-methylglutamine, and γ-methylglutamine in phosphate solution at pH 7.8 and in N hydrochloric acid was studied.

5. The preparation and certain properties of sodium α-ketoadipamate, barium α-keto-*N*-methyladipamate, barium α-keto-*N*-dimethylglutaramate, and barium α-keto-*dl*-γ-methylglutaramate are given.

6. A procedure is described for the preparation of pure glutamine, homoglutamine, and α-methylglutamine, free of the corresponding α-aminodicarboxylic acids.

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